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Transforming growth factor-β1 regulation of growth zone chondrocytes is mediated by multiple interacting pathways

Enrique Rosado [a,b], Zvi Schwartz [a,c,d], Victor L. Sylvia [d], David D. Dean [d], Barbara D. Boyan [a,d,e,*]

[a] *Department of Periodontics, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*
[b] *Department of Periodontics, Wilford Hall Medical Center, Lackland Air Force Base, TX 78236, USA*
[c] *Department of Periodontics, Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem 91010, Israel*
[d] *Department of Orthopaedics, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*
[e] *Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*

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Abstract

Transforming growth factor beta 1 (TGF-β1) affects growth plate chondrocytes through Smad-mediated mechanisms and has been shown to increase protein kinase C (PKC). This study determined if PKC mediates the physiological response of rat costochondral growth zone (GC) chondrocytes to TGF-β1; if the physiological response occurs via type II or type III TGF-β receptors, and, if so, which receptor mediates the increase in PKC; and the signal transduction pathways involved. Treatment of confluent GC cells with TGF-β1 stimulated [^{3}H]thymidine and [^{35}S]sulfate incorporation as well as alkaline phosphatase (ALPase) and PKC specific activities. Inhibition of PKC with chelerythrine, staurosporine, or H-7 caused a dose-dependent decrease in these parameters, indicating that PKC signaling was involved. TGF-β1-dependent PKC and the physiological response of GC cells to TGF-β1 was reversed by anti-type II TGF-β receptor antibody and soluble type II TGF-β receptor, showing that TGF-β1 mediates these effects through the type II receptor. The increase in [^{3}H]thymidine incorporation and ALPase specific activity were also regulated by protein kinase A (PKA) signaling, since the effects of TGF-β1 were partially blocked by the PKA inhibitor H-8. The mechanism of TGF-β1 activation of PKC is through phospholipase A_2 (PLA_2) and not through phospholipase C (PLC). Arachidonic acid increased PKC in control cultures and was additive with TGF-β1. Prostanoids are required, as indomethacin blocked the effect of TGF-β1, and Cox-1, but not Cox-2, is involved. TGF-β1 stimulates prostaglandin E_2 (PGE_2) production and exogenous PGE_2 stimulates PKC, but not as much as TGF-β1, suggesting that PGE_2 is not sufficient for all of the prostaglandin effect. In contrast, TGF-β1 was not regulated by diacylglycerol; neither dioctanoylglycerol (DOG) nor inhibition of diacylglycerol kinase with R59022 had an effect. G-proteins mediate TGF-β1 signaling at different levels in the cascade. TGF-β1-dependent increases in PGE_2 levels and PKC were augmented by the G protein activator GTPγS, whereas inhibition of G-protein activity via GDPβS, pertussis toxin, or cholera toxin blocked stimulation of PKC by TGF-β1, indicating that both G_i and G_s are involved. Inhibition of PKA with H-8 partially blocked TGF-β1-dependent PKC, suggesting that PKA inhibition on the physiological response was via PKA regulation of PKC signaling. This indicates that multiple interacting signaling pathways are involved: TGF-β1 stimulates PLA_2 and prostaglandin release via the action of Cox-1 on arachidonic acid. PGE_2 activates the EP2 receptor, leading to G-protein-dependent activation of PKA. PKA signaling results in increased PKC activity and PKC signaling regulates proliferation, differentiation, and matrix synthesis.

Keywords: Chondrocyte culture; Transforming growth factor-β1; 1,25-$(OH)_2D_3$; Protein kinase C; Alkaline phosphatase; Signal transduction

1. Introduction

Transforming growth factor beta (TGF-β) is an important regulator of chondrocyte proliferation and differentiation that was originally purified from demineralized bone based on its ability to stimulate the differentiation of neonatal rat muscle cells into chondrocytes [1–3]. TGF-β also regulates committed chondrocytes, including those in the endochon-

* Corresponding author. Department of Orthopaedics, MSC 7774, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA. Tel.: +1-210-567-6326; fax: +1-210-567-6295.
E-mail address: BoyanB@uthscsa.edu (B.D. Boyan).

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dral pathway [4–6], and TGF-β1, TGF-β2, and TGF-β3 are expressed in the resting, proliferating, and maturing zones of the rat epiphyseal growth plate [7]. Rat costochondral cartilage cells are particularly sensitive to TGF-β1 [6,8]. These cells respond to the growth factor in a concentration-dependent manner. At levels as low as 0.22 ng/ml, costochondral cartilage cells exhibit maximal responses with respect to differentiation; at higher concentrations of 0.88 ng/ml, proliferation is affected. Even proliferation is regulated at levels of the growth factor that are significantly lower than are required for osteoblasts to exhibit the same effect [9,10]. Rat articular chondrocytes also exhibit increased proliferation in response to low concentrations of TGF-β1 [11].

The mechanisms by which TGF-β exerts its effects on growth plate chondrocytes are beginning to be elucidated. The high degree of sensitivity of rat chondrocytes to TGF-β1 and the narrow concentration ranges eliciting specific responses in costochondral growth plate chondrocytes suggest that complex signaling pathways may be involved. There is considerable evidence that the physiological response of cells to TGF-β1 is mediated by the type II TGF-β1 receptor [12–16] and that the biological signal of TGF-β1 is conveyed through Smad proteins [17,18]. TGF-β receptors I and II are expressed in resting, proliferating, and maturing zones of the rat epiphyseal growth plate [7], suggesting that they may also function in the costochondral cartilage as well. Upon binding to TGF-β1, the type II receptor associates with and activates the type I receptor; Smads 2 and 3 are phosphorylated and translocate into the nucleus, where they act as transcription factors [19].

It is becoming increasingly clear that Smads may not be solely responsible for the entire effect of TGF-β1. We have shown that TGF-β1 causes a dose-dependent increase in protein kinase C (PKC) in costochondral chondrocytes [20] and that PKC mediates some, but not all, of the biological responses of costochondral cartilage resting zone chondrocytes to TGF-β1. Whether this is also true for cells from the prehypertrophic and upper hypertrophic zones (growth zone) of the growth plate is not known, nor is it known if it is due to the action of TGF-β1 on the type II receptor.

There are a number of reasons why TGF-β1 action may differ in resting zone and growth zone chondrocytes. TGF-β1 causes an increase in PKCα specific activity that is maximal at 12 h and requires gene expression and protein synthesis in both resting zone and growth zone chondrocytes, but the time course of the response differs between the two states of endochondral maturation [20]. In resting zone cells, the increase in PKC occurs earlier and remains elevated over a longer period of time than is seen in growth zone cells. In both cell types, the increase in enzyme activity is due to new cytosolic PKC and only minimally to the translocation of existing PKC to the plasma membrane. There are some differences in the mechanisms involved in TGF-β1-dependent PKC activation in resting zone and growth zone cells, however. Inhibition of phosphatidylinositol-specific phospholipase C (PI-PLC) caused a small, but significant, dose-dependent increase in PKC in growth zone cells, and inhibition of tyrosine kinase caused a similar increase in PKC in resting zone cells [20].

Other signaling pathways may also be involved as well. Whereas the effect of TGF-β1 on proteoglycan production in resting zone chondrocytes is PKC-dependent, inhibition of PKC does not affect the TGF-β1-stimulated increase in proliferation or alkaline phosphatase (ALPase) activity. One possibility is that protein kinase A (PKA) mediates at least part of the response to TGF-β1. Studies examining the physiological effects of TGF-β1 in rat articular chondrocytes support this hypothesis [11]. TGF-β1 causes a rapid increase in c-*fos* expression in these cells that is followed by an increase in [^{3}H]thymidine incorporation. The PKC inhibitor H7 blocks the effect, implicating PKC in the mechanism. However, these investigators used concentrations of H-7 that also block PKA [21], so it is not clear whether PKC or PKA was involved in the rapid response to the growth factor. Specific inhibition of PKA also inhibits the stimulatory effect of TGF-β1 on proliferation and ALPase in costochondral cartilage resting zone cells [22].

Both PKC and PKA signaling can lead to activation of MAP kinase [23,24]. TGF-β1 has been shown to activate the Ras-Raf-MEK-ERK MAP kinase pathway [25–27], and activation of MAP kinase is via PKC [28]. These observations suggest that receptor binding may regulate the physiology of growth plate chondrocytes through PKC and PKA, in addition to using Smad proteins. It is not known which receptor is responsible for the TGF-β1 effect on PKC or on PKC-dependent physiological responses.

Phospholipase A_2 (PLA_2), arachidonic acid, and prostaglandin E_2 (PGE_2) may also play a role in the mechanism by which TGF-β1 regulates PKC in growth zone chondrocytes. We recently showed that TGF-β1 causes an increase in PGE_2 production in resting zone cells, and this is further increased by PKC inhibition [29]. The increase in PGE_2 was due to an increase in PLA_2 activity rather than an increase in arachidonic acid metabolism. In addition, exogenous arachidonic acid inhibited TGF-β1-dependent stimulation of PKC. Whether this is also true for growth zone chondrocytes is not known, but there are a number of reasons to expect it not to be. Arachidonic acid decreases PKC in resting zone cells, but increases enzyme activity in growth zone cells [30]. PGE_2 signals through its EP1 receptor to decrease PKC in resting zone cells [31], whereas in growth zone cells, PGE_2 stimulates PKC [30]. Moreover, many of the physiological responses of resting zone cells and growth zone cells to TGF-β1 differ [8,32], supporting the hypothesis that different receptor-mediated pathways are involved.

This study tested the hypothesis that TGF-β1 acts on costochondral growth zone (GC) chondrocytes via the TGF-β1 type II receptor. The physiological responses of the cells are mediated by PKC and PKA in a mechanism that involves PLA_2 through the release of arachidonic acid and prostaglandin production. Moreover, regulation of PKC by

TGF-β1 and the signaling pathways responsible depend on the maturation state of the responding chondrocyte.

2. Materials and methods

2.1. Reagents

The following chemicals were purchased from Sigma Chemical (St. Louis, MO): PGE_2, indomethacin (a general cyclooxygenase inhibitor), and reagents for the ALPase assay. The following chemicals were purchased from Calbiochem (San Diego, CA): 1,2-dioctanoyl-*sn*-glycerol (DOG), R59022 (diacylglycerol kinase inhibitor), chelerythrine, staurosporine, and H-7 (PKC inhibitors), H-8 (PKA inhibitor), arachidonic acid, pertussis toxin (G_i inhibitor), choleratoxin (G_s inhibitor), GDPβS (general G-protein inhibitor), GTPγS (G-protein activator), resveratrol (Cox-1 inhibitor), and NS-398 (Cox-2 inhibitor). PKC assay reagents and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent [33] obtained from Pierce Chemical (Rockford, IL). Recombinant human TGF-β1, anti-TGF-β1 receptor II and receptor III antibodies, and soluble TGF-β1 receptor II and receptor III were obtained from R&D Systems (Minneapolis, MN). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). The PGE_2 radioimmunoassay kit, [^{3}H]thymidine, [^{35}S]sulfate, and [^{32}P]ATP were obtained from NEN-DuPont (Boston, MA).

2.2. Chondrocyte cultures

The rat costochondral chondrocyte culture system used in this study has been described in detail previously [34]. Cells were derived from the growth zone (prehypertrophic and upper hypertrophic cell zones) of costochondral cartilage from 125-g male Sprague-Dawley rats (Harlan, Indianapolis, IN) and cultured in DMEM containing 10% FBS, 50 μg/ml vitamin C, and antibiotics. Confluent, fourth passage cells were used for all experiments. The characteristics of these cells have been well described [35,36]. Although they express mRNA for type X collagen based on RT-PCR (unpublished data), they do not produce type X collagen based on SDS-PAGE [37]. The cells produce type II collagen, synthesize sulfated glycosaminoglycans, and form cartilage nodules when implanted in nude mouse thigh muscle.

2.3. Role of TGF-β1 receptors in physiologic response of growth zone cells to TGF-β1

To determine whether the response of growth zone cells to TGF-β1 involves the type II or type III TGF-β1 receptor, cultures were incubated with 0.11, 0.22, or 0.88 ng/ml TGF-β1±0.5, 1 or 2 μg/ml anti-type II receptor or anti-type III receptor antibodies, or with 25, 50 or 100 μg/ml soluble type II or III receptors for 24 h. Changes in [^{3}H]thymidine incorporation, proteoglycan sulfation, and ALPase specific activity were assessed. We did not specifically examine the role of the type I receptor since antibodies to this receptor were not available. We did not include cultures treated with nonspecific IgG_1 because previous studies failed to show an effect on any of the parameters we examined [38].

2.3.1. [^{3}H]thymidine incorporation

DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously [39]. Quiescence was induced by incubating cultures for 48 h in DMEM containing 1% FBS. The medium was then replaced for 24 h with DMEM containing 1% FBS, vehicle ± TGF-β1, and the appropriate concentration of soluble receptor or anti-receptor antibody. Two hours prior to harvest, [^{3}H]thymidine was added. Radioactivity in TCA-precipitable material was measured by liquid scintillation spectroscopy.

2.3.2. [^{35}S]sulfate incorporation

Proteoglycan synthesis was assessed by measuring [^{35}S]sulfate incorporation using a modification of the method of O'Keefe et al. [40] as described previously [41,42]. At confluence, fresh medium containing vehicle ± TGF-β1 ± soluble receptors or anti-receptor antibodies was added to the cells and the incubation continued for an additional 24 h. Four hours prior to harvest, 50 μl DMEM containing 18 μCi/ml [^{35}S]sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [^{35}S]sulfate incorporated determined by liquid scintillation spectrometry. The protein content was determined using the BCA protein assay and the data expressed as disintegrations per minute per milligram protein in the cell layer.

2.3.3. ALPase activity

ALPase [orthophosphoric monoester phosphohydrolase, alkaline (EC 3.1.3.1)] was measured as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2 [43]. Enzyme activity was assayed in lysates of the cell layer, as well as in isolated matrix vesicle and plasma membranes as previously described [44]. Cultures were treated with 0.11, 0.22, or 0.88 ng/ml TGF-β1 ± soluble receptors or anti-receptor antibodies.

2.4. Role of PKC in physiologic response of growth zone cells to TGF-β1

To determine whether the response of growth zone cells to TGF-β1 involves PKC, cultures were incubated with 0.11, 0.22, or 0.88 ng/ml TGF-β1 ± PKC inhibitors. We used 1 and 10 μM chelerythrine as an inhibitor of PKC because of its relative specificity for the enzyme [45]. In some

experiments, cells were cultured with 0.01 or 0.1 μM staurosporine [46] or 1, 5, or 10 μM H-7 [21] to inhibit PKC. All three inhibitors have been shown to cause dose-dependent decreases in PKC activity of growth zone cell cultures [47]. [^{3}H]thymidine incorporation, [^{35}S]sulfate incorporation, and ALPase were measured as described above.

2.5. PGE_2 production

Because PGE_2 is a regulator of growth zone chondrocytes [48] and is involved in the rapid activation of PKC in these cells in response to other mediators [49], we examined whether TGF-β1 increases PGE_2 production as well. PGE_2 production by growth zone chondrocyte cultures treated with TGF-β1 was assessed by using a radioimmunoassay kit (NEN DuPont) as described previously [50]. PGE_2 has been shown to regulate physiologic responses of growth zone chondrocytes by signaling through the EP1 receptor [51], which signals through the Gq/PLC pathway to activate PKC [52]. To clarify whether the effect of TGF-β1 on PGE_2 production was before or after G-protein activation or PKC activation, confluent cultures were treated for 24 h with 0.22 ng/ml TGF-β1 in the presence or absence of 1, 10 or 100 μM GTPγS (general G-protein activator) or chelerythrine (PKC inhibitor).

2.6. Role of PKA

PGE_2 exerts its effects on growth zone cells via the EP1 receptor through both PKC- and PKA-dependent mechanisms [51]. To assess the role of PKA in cell response to TGF-β1, growth zone chondrocytes were incubated with TGF-β1±1, 5, or 10 μM H-8 [21]. H-8 inhibits PKA with an IC_{50} of 1.2 μM. In contrast, the IC_{50} for PKC is 15 μM, and the IC_{50} for MLCK is 68 μM [53], well above the concentrations used in this study. [^{3}H]thymidine incorporation was examined in cultures treated with 0.88 ng/ml TGF-β1, and ALPase specific activity was measured in cultures treated with 0.22 ng/ml TGF-β1 based on prior studies, indicating that maximal effects of TGF-β1 on these parameters occur at these concentrations [6,54]. Assays were performed as described above.

2.7. TGF-β1-dependent regulation of PKC

2.7.1. PKC specific activity

PKC specific activity was assayed as described previously [20]. Cell layer lysates containing equivalent amounts of protein were mixed for 20 min with a lipid preparation containing phorbol-12-myristate-13-acetate, phosphatidylserine, and Triton X-100 mixed micelles to provide the necessary cofactors and conditions for optimal enzyme activity [55]. To this mixture, a high-affinity myelin basic protein peptide and [^{32}P]ATP (25 μCi/ml) were added to a final assay volume of 50 μl. Following a 10-min incubation at 30 °C, samples were spotted onto phosphocellulose discs, which were then washed twice with 1% phosphoric acid and once with distilled water to remove unincorporated label prior to placement in a scintillation counter.

2.7.2. Effect of arachidonic acid, prostaglandins, and PGE_2

To assess the role of arachidonic acid in TGF-β1-dependent PKC stimulation, confluent cultures were treated for 12 h with vehicle alone or with 0.22 ng/ml TGF-β1 in the presence or absence of 1, 10 or 100 μM arachidonic acid. Control cultures were treated with TGF-β1+the arachidonic acid vehicle (0.02% ethanol in DMEM). To determine if prostaglandin mediates the effects of TGF-β1 on PKC, growth zone chondrocytes were incubated in the presence or absence of 0.1, 1, or 10 μM indomethacin±0.22 ng/ml TGF-β1 for 12 h. Indomethacin blocks both Cox-1 and Cox-2 [56]. The relative contributions of Cox-1 and Cox-2 were determined using resveratrol (0.1, 1, and 10 μM) and NS-398 (0.1, 1, and 10 μM) to inhibit the constitutive and inducible forms of the enzyme, respectively. The specific role of PGE_2 was determined by treating the cultures with vehicle±0.22 ng/ml TGF-β1±15, 60, or 240 pg/ml PGE_2.

2.7.3. Effect of diacylglycerol

Diacylglycerol activates PKC in a number of cells, including growth zone chondrocytes [47]. This is generally a rapid effect due to rapid Gq-dependent activation of PLC [47]. DAG then binds to PKC, activating translocation of PKC to the plasma membrane [57]. Our previous studies showed that PLC was not involved in the mechanism of TGF-β1-dependent PKC activation [20]. Thus, if diacylglycerol was involved, it must be generated through another pathway. Diacylglycerol can also be produced through phospholipase D (PLD) via a mechanism involving PLD2 [58], but this pathway may not occur with the same rapidity as via PLC. To determine if diacylglycerol is involved in TGF-β1 action, we examined the effects of DOG, a cell-permeable form of the PKC activator [57], and the diacylglycerol kinase inhibitor R59022, which maintains elevated diacylglycerol levels [59]. R59022 blocks the conversion of diacylglycerol species to phosphatidic acid and is relatively promiscuous about the source of the diacylglycerol [59–61]. We did not use butan-1-ol to block PLD [62] because butanol is toxic to the cells. Cultures were treated with vehicle±0.11 or 0.22 ng/ml TGF-β1 in the presence or absence of 1, 10, or 100 μM DOG or 5, 10, or 50 μM R59022. Control cultures were treated with TGF-β1±vehicle (0.02% ethanol in DMEM). PKC activity was measured at 12 h.

2.7.4. Direct effect of TGF-β1 on membrane-associated PKC

PKC activity of membranes increases in response to a stimulus due to translocation of the activated cytosolic enzyme to the membrane [63]. However, previous studies in our laboratory showed that TGF-β1-dependent increases

in PKC are not due to translocation [20]. To determine whether TGF-β1 regulates PKC activity by direct action on membrane-associated enzyme, isolated plasma membranes and matrix vesicles were incubated directly with TGF-β1. Both membrane fractions have been shown previously to contain PKCα and PKCζ [64]; PKCα predominates in plasma membranes, whereas PKCζ predominates in matrix vesicles. Because matrix vesicles are extracellular, they are right side out when they are isolated. They do not contain RNA or DNA, so new gene expression or protein synthesis cannot occur. Therefore, any effect of TGF-β1 on PKC is by direct action on the membrane.

For these experiments, confluent, fourth passage growth zone cells were released from their matrix by trypsin digestion and collected by centrifugation. Plasma membranes were isolated by differential and sucrose density gradient centrifugation of homogenized cells [44,65]. Matrix vesicles were isolated by differential centrifugation of the trypsin-digested matrix [66]. Following assay for protein content [33], plasma membranes or matrix vesicles were suspended in 0.9% NaCl and frozen at −70 °C. Matrix vesicles isolated in this manner typically exhibit greater than two-fold enrichment of ALPase specific activity when compared with the plasma membranes and have a transmission electron microscopic appearance consistent with matrix vesicles in vivo [66]. Matrix vesicles or plasma membranes (10 μg protein in 0.9% NaCl containing 10% FBS) were incubated in the absence (vehicle only) or presence of a final concentration of 0.11, 0.22, or 0.88 ng/ml TGF-β1 for either 9, 90, or 270 min at 37 °C as described previously [64]. Following incubation, samples were assayed for PKC activity.

2.8. Statistical analysis

Unless otherwise noted, the data presented below are from one of three or more independent experiments, all showing comparable results. Each data point represents the

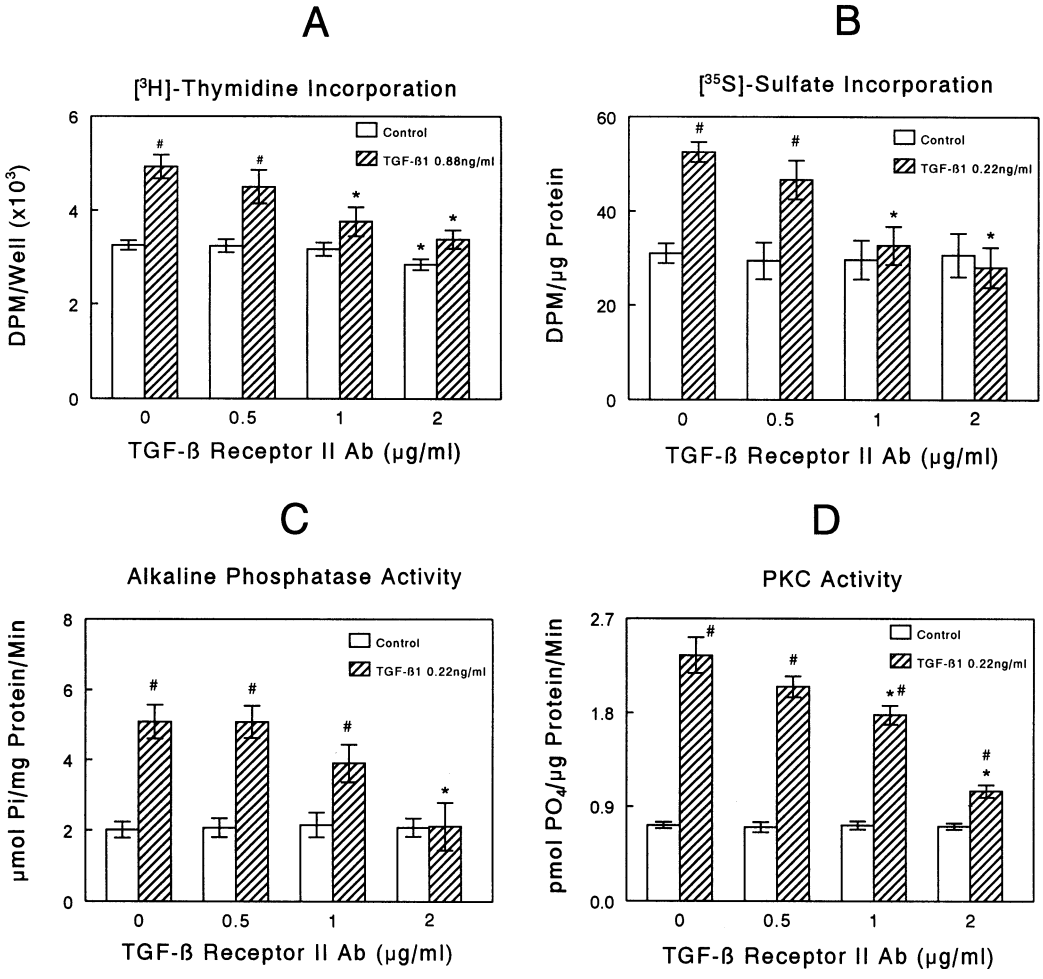

Fig. 1. Effect of TGF-β1 receptor II antibody on TGF-β1-induced [^{3}H]thymidine incorporation (A); [^{35}S]sulfate incorporation (B); alkaline phosphatase activity (C); and PKC activity (D). Confluent growth zone cells were treated with control media or TGF-β1 in the absence and presence of TGF-β1 receptor II antibody as described in Materials and methods. Values are the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results. *$P<0.05$, vs. no treatment with antibody; $^{\#}P<0.05$, vs. no treatment with TGF-β1.

mean ± S.E. for six cultures (N=6). For membrane PKC assays, each data point is the mean ± S.E. for six membrane preparations, where each preparation was derived from one T-150 flask of cells. Data were analyzed by ANOVA. Statistical significance was determined using Bonferroni's modification of Student's t-test, with $P<0.05$ being considered significant.

3. Results

3.1. Role of TGF-β1 receptors in physiologic response of growth zone cells to TGF-β1

TGF-β1 mediates its effects on growth zone chondrocytes via its type II receptor. Antibodies to the TGF-β1 type II receptor (Fig. 1), as well as soluble type II receptor (Fig. 2), caused a dose-dependent decrease in TGF-β1-stimulated [^{3}H]thymidine incorporation (Figs. 1A and 2A), proteoglycan sulfation (Figs. 1B and 2B), ALPase specific activity (Figs. 1C and 2C), and PKC specific activity (Figs. 1D and 2D). The inhibitory effect of the soluble receptor on the response of growth zone cells to TGF-β1 was only partial with respect to [^{35}S]sulfate incorporation and PKC activity (Fig. 2B,D). Similarly, TGF-β1-dependent increases in PKC were elevated over control levels, even at the highest concentration of antibody used (Fig. 1D). Neither the antibody nor the soluble receptor affected these parameters in control cultures. In contrast to TGF-β receptor II antibody, TGF-β receptor III antibody had no significant effect on PKC specific activity in either control cultures or cultures treated with 0.22 ng/ml TGF-β1 (data not shown).

3.2. Role of PKC in cell response to TGF-β1

The physiological response to TGF-β1 was PKC-dependent (Fig. 3). Chelerythrine reduced the TGF-β1-dependent increase on [^{3}H]thymidine incorporation (Fig. 3A), [^{35}S]sulfate incorporation (Fig. 3B), and ALPase specific activity (Fig. 3C). The PKC-dependent effects of TGF-β1 on [^{3}H]thymidine incorporation were confirmed using the PKC inhibitors staurosporine (data not shown) and

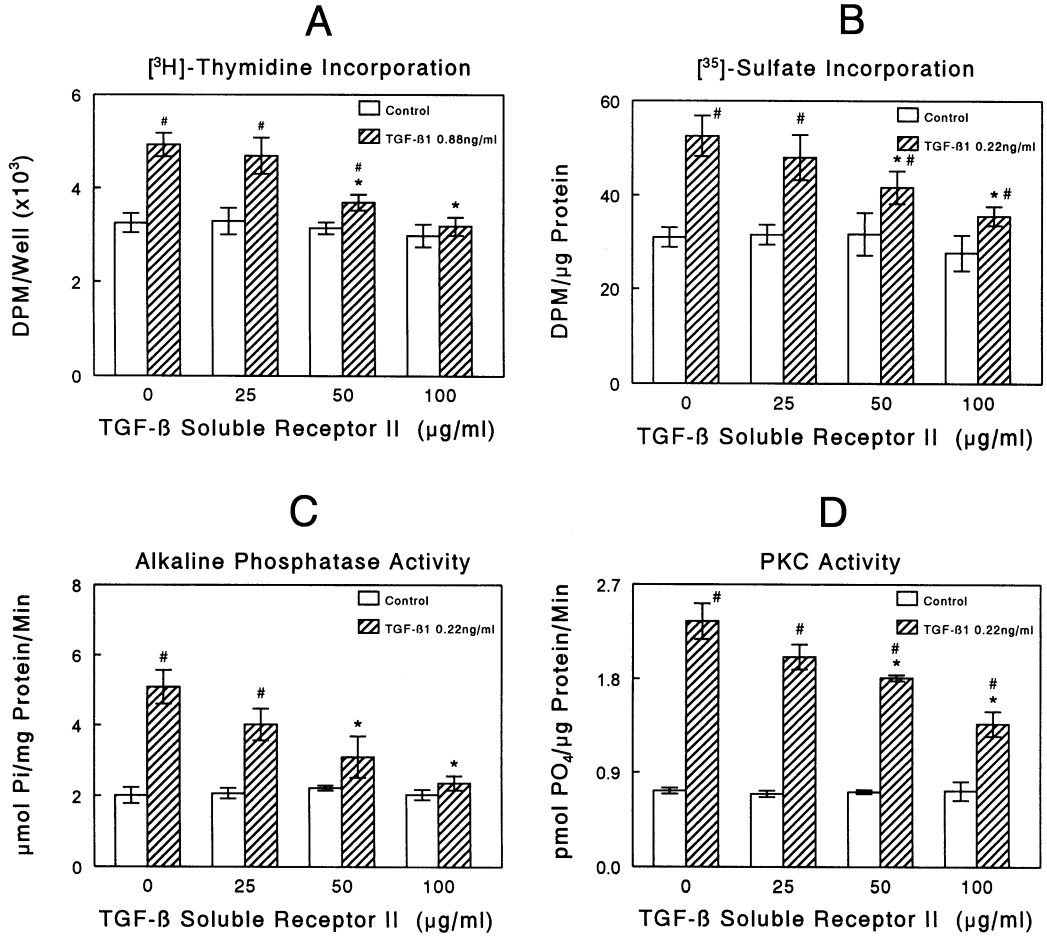

Fig. 2. Effect of TGF-β1 soluble type II receptor on TGF-β1-induced [^{3}H]thymidine incorporation (A); [^{35}S]sulfate incorporation; (B); alkaline phosphatase activity (C); and PKC activity (D). Confluent growth zone cells were treated with control media or TGF-β1 in the absence and presence of 25, 50 or 100 µg/ml soluble type II receptor as described in Materials and methods. Each data point is the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results. *$P<0.05$, vs. no treatment with soluble receptor; $^{\#}P<0.05$, vs. no treatment with TGF-β1.

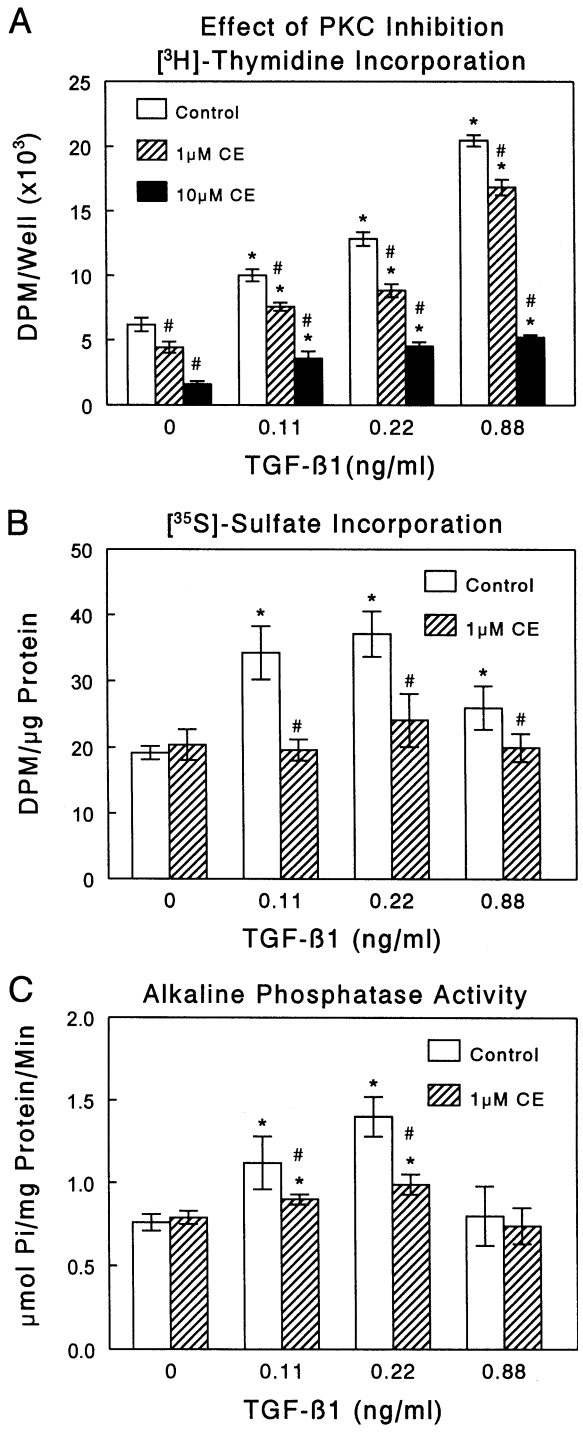

Fig. 3. Effect of PKC inhibitor chelerythrine on TGF-β1-induced [^{3}H]thymidine incorporation (A); [^{35}S]sulfate incorporation (B); and alkaline phosphatase specific activity (C). Confluent growth zone cells were treated for 24 h with control media or 0.11, 0.22, or 0.88 ng/ml TGF-β1 in the presence and absence of 1 or 10 μM chelerythrine (CE). Values are the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results. *$P<0.05$, vs. no treatment with TGF-β1; $^{\#}P<0.05$, vs. no treatment with PKC inhibitor.

Table 1
Effect of protein kinase inhibitors on TGF-β1-induced [^{3}H]thymidine incorporation

Effector	[^{3}H]thymidine incorporation, DPM/well ($\times 10^4$)
Control	1.34±0.03
PKC inhibitor (H-7)	
1 μM	1.36±0.02
5 μM	1.38±0.03
10 μM	1.40±0.04
PKA inhibitor (H-8)	
1 μM	1.35±0.06
5 μM	1.50±0.04
10 μM	1.37±0.02
TGF-β1 (0.88 ng/ml)	4.24±0.20*
TGF-β1+1 μM H-7	2.85±0.46*,#
TGF-β1+5 μM H-7	2.20±0.71*,#
TGF-β1+10 μM H-7	1.40±0.37*,#
TGF-β1+1 μM H-8	3.82±0.36*
TGF-β1+5 μM H-8	3.38±0.36*,#
TGF-β1+10 μM H-8	3.02±0.29*,#

Confluent growth zone cells were treated for 24 h with control media or 0.88 ng/ml TGF-β1 in the absence and presence of 1, 5, and 10 μM H-7, a PKC inhibitor, or H-8, a PKA inhibitor. At harvest, [^{3}H]thymidine incorporation in the cell layer was measured. Each value is the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results.

* $P<0.05$, treatment vs. control.

# $P<0.05$, TGF-β1+inhibitor vs. TGF-β1 alone.

H-7 (Table 1). Similarly, H-7 blocked the effects of TGF-β1 on ALPase (Table 2).

Part of the physiological response to TGF-β1 was also mediated via a PKA-dependent mechanism. H-8, a specific

Table 2
Effect of protein kinase inhibitors on TGF-β1-induced alkaline phosphatase specific activity

Effector	Alkaline phosphatase specific activity (μmol Pi/mg protein/min)
Control	6.92±0.43
PKC inhibitor (H-7)	
1 μM	7.37±0.30
5 μM	6.78±0.29
10 μM	5.56±0.67*
PKA inhibitor (H-8)	
1 μM	7.07±0.62
5 μM	6.91±0.71
10 μM	5.81±0.53*
TGF-β1 (0.22 ng/ml)	11.79±0.60*
TGF-β1+1 μM H-7	8.67±0.50*,#
TGF-β1+5 μM H-7	7.94±0.80#
TGF-β1+10 μM H-7	7.31±0.55#
TGF-β1+1 μM H-8	9.45±0.22*,#
TGF-β1+5 μM H-8	8.64±0.19*,#
TGF-β1+10 μM H-8	8.16±0.72*,#

Confluent growth zone cells were treated for 24 h with control media or 0.22 ng/ml TGF-β1 in the absence and presence of 1, 5, and 10 μM H-7, a PKC inhibitor, or H-8, a PKA inhibitor. At harvest, alkaline phosphatase specific activity in the cell layer was measured. Each value is the mean ± S.E. of six independent cultures.

* $P<0.05$, treatment vs. control.

# $P<0.05$, TGF-β1+inhibitor vs. TGF-β1 alone.

inhibitor of PKA reduced TGF-β1-stimulated [^{3}H]thymidine incorporation by approximately 30% (Table 1). H-8 also partially blocked the TGF-β1-stimulated increase in ALPase specific activity (Table 2).

The production of PGE_2 by growth zone chondrocyte cultures was increased by TGF-β1 in a biphasic manner (Fig. 4A). Significant increases in PGE_2 production were observed for cultures treated with 0.12, 0.22, and 0.5 ng/ml TGF-β1. PGE_2 production varied with time of treatment (Fig. 4B). In control cultures, PGE_2 levels were increased by 12 h and remained elevated at 24 h. Treatment with TGF-β1 (0.22 ng/ml) caused a robust increase in PGE_2 production at all times examined. At 3 h, there was a 400% increase; at 6 h, there was a 300% increase; at 12 h, the increase was more than 100%; and at 24 h, the increase was slightly more than 50% over control cultures.

The effect of TGF-β1 on PGE_2 production was G-protein-dependent (Fig. 4C). The G-protein activator GTPγS had no effect on basal PGE_2 production, but increased TGF-β1-induced PGE_2 production in a dose-dependent manner. Maximal increases were observed in cultures treated with 10 μM GTPγS. In contrast, PGE_2 production in response to TGF-β1 was not mediated by PKC. Inhibition of PKC with 1 μM chelerythrine had no effect on PGE_2 production in either control cultures or cultures treated with TGF-β1 (data not shown). Interestingly, there was a slight, but statistically significant, decrease in PGE_2 in control cultures at 10 and 100 μM chelerythrine.

3.3. Mechanism of action of TGF-β1 on PKC activity

TGF-β1 regulates PKC activity through a PLA_2-dependent signaling pathway. Arachidonic acid, the product of PLA_2 action, increased PKC activity in growth zone chondrocytes, and at the highest concentration examined, the stimulatory effect of the fatty acid was additive with that of TGF-β1 (Table 3). Part of the effect of arachidonic acid may be due to its metabolism by cyclooxygenase and production of prostaglandin. Inhibition of cyclooxygenase with indomethacin, which inhibits both Cox-1 and Cox-2, reduced TGF-β1-induced PKC activity in a dose-dependent manner (Fig. 5A). PKC activity in control cultures was reduced by 34% at the highest concentration used, and TGF-β1-induced activity was reduced by 61%. The cyclooxygenase responsible was Cox-1, since the Cox-1 inhibitor, resveratrol, significantly reduced basal PKC activity at 10 μM by 30%, and reduced TGF-β1-induced activity at 1 and 10 μM by up to 60% (Fig. 5B). The Cox-2 inhibitor NS-398 had no effect on PKC activity in either control or TGF-β1-treated cultures (Fig. 5C).

Some of the prostaglandin-mediated effect may be due to PGE_2. PGE_2 dose-dependently increased PKC activity in control cultures (Table 3). Further, the effect due to PGE_2 was less than half as robust as the effect due to TGF-β1. Moreover, PGE_2 had no effect on the increase in activity elicited by TGF-β1.

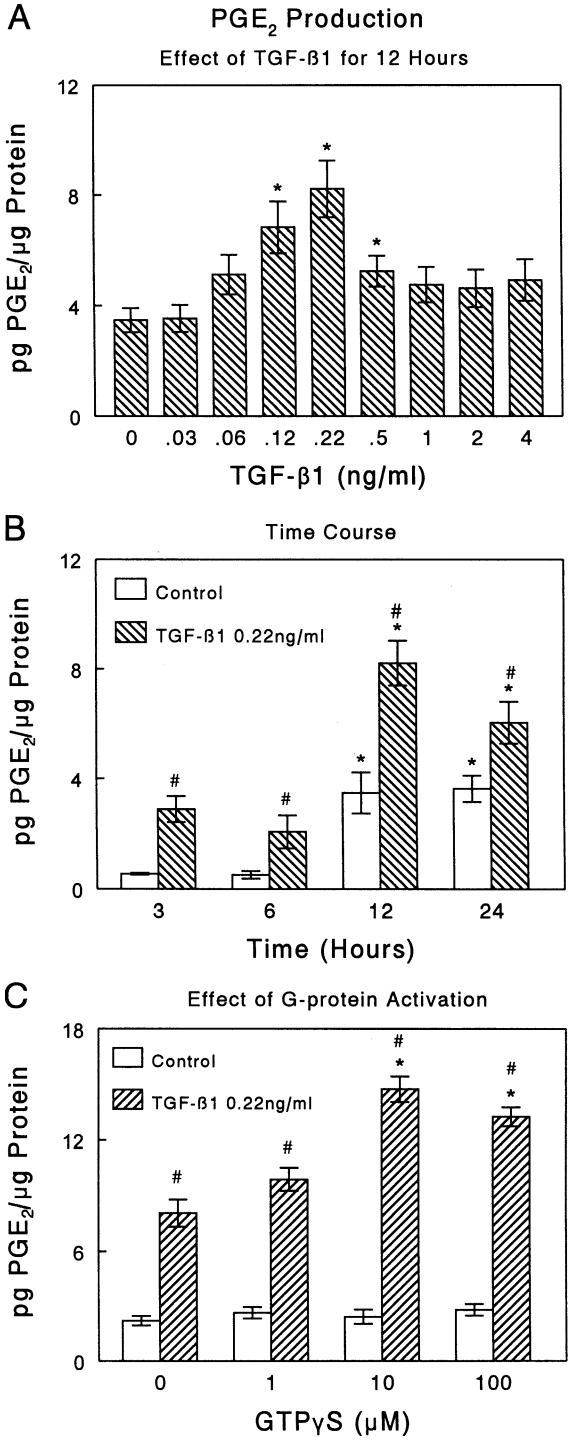

Fig. 4. Effect of TGF-β1 on PGE_2 production. Confluent growth zone cells were treated for 12 h with control media or media containing 0.03–4 ng/ml TGF-β1 (Panel A). Alternatively, cells were treated with control media or media containing 0.22 ng/ml TGF-β1 for 3, 6, 12, or 24 h (Panel B). The role of G-protein activation was examined using the G-protein activator GTPγS. Confluent growth zone cells were treated for 12 h with control media or 0.22 ng/ml TGF-β1 in the presence and absence of 1, 10, or 100 μM GTPγS (Panel C). At harvest, cultures were assayed for PGE_2 release into the media. Values are the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results. *$P<0.05$, vs. control (Panel A); 3 h (Panel B); or no activator (Panel C). #$P<0.05$, vs. not treated with TGF-β1.

Table 3
Effect of arachidonic acid and PGE_2 on TGF-β1-induced PKC specific activity

Effector	Protein kinase C specific activity (pmol PO_4/μg protein/min), 12 h
Control	0.48±0.04
Arachidonic acid (AA)	
1 μM	0.49±0.03
10 μM	0.63±0.04*
100 μM	0.86±0.06*,+
PGE_2	
0.015 ng/ml	0.61±0.02*
0.06 ng/ml	0.69±0.06*,^
0.24 ng/ml	0.74±0.06*,^
TGF-β1 (0.22 ng/ml)	1.61±0.30*
TGF-β1+1 μM AA	1.77±0.36*
TGF-β1+10 μM AA	1.84±0.09*
TGF-β1+100 μM AA	2.49±0.39*,#
TGF-β1+0.015 ng/ml PGE_2	1.59±0.11*
TGF-β1+0.06 ng/ml PGE_2	1.50±0.14*
TGF-β1+0.24 ng/ml PGE_2	1.60±0.20*

Confluent growth zone cells were treated for 12 h with control media or 0.22 ng/ml TGF-β1 in the absence and presence of 1, 10, or 100 μM arachidonic acid or 0.015, 0.06, or 0.24 ng/ml PGE_2. At harvest, PKC-specific activity in the cell layer was determined. Each value is the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results.

* $P<0.05$, vs. control.
+ $P<0.05$, vs. 1 μM AA.
^ $P<0.05$, vs. 0.015 ng/ml PGE_2.
# $P<0.05$, vs. TGF-β1 alone.

The effect of TGF-β1 on PKC does not involve diacylglycerol, indicating that the PLC and PLD signaling pathways do not play a role. DOG caused a dose-dependent increase in PKC activity at 90 min, as did treatment of the cells with the diacylglycerol kinase inhibitor R59022, but TGF-β1 had no effect at that time (data not shown). In cultures treated with TGF-β1+DOG or R59022, only the effect of diacylglycerol was evident. In contrast, at 12 h, neither DOG nor R59022 affected PKC, and neither agent altered the stimulatory effect of TGF-β1 (data not shown).

G-proteins are involved in the action of TGF-β1 on PKC activity in growth zone chondrocytes. Pertussis toxin (Fig. 6A), which targets G_i-proteins, and cholera toxin (Fig. 6B), which targets G_s-proteins, both increased PKC activity slightly in control cultures, but reduced TGF-β1-induced PKC activity in a dose-dependent manner. At the highest concentrations examined, both inhibitors completely blocked the stimulatory effect of TGF-β1 on PKC. These observations were confirmed using the general G-protein inhibitor GDPβS (Fig. 6C). Whereas GDPβS had no effect on PKC in control cultures, it caused a dose-dependent decrease in TGF-β1-stimulated PKC. At the highest concentration used, the effect of TGF-β1 was completely blocked. In contrast, the G-protein activator GTPγS caused a dose-dependent increase in TGF-β1-stimulated PKC activity (Fig. 6D).

PKA is involved in TGF-β1 regulation of PKC activity in growth zone cells. The PKA inhibitor H-8 caused a small,

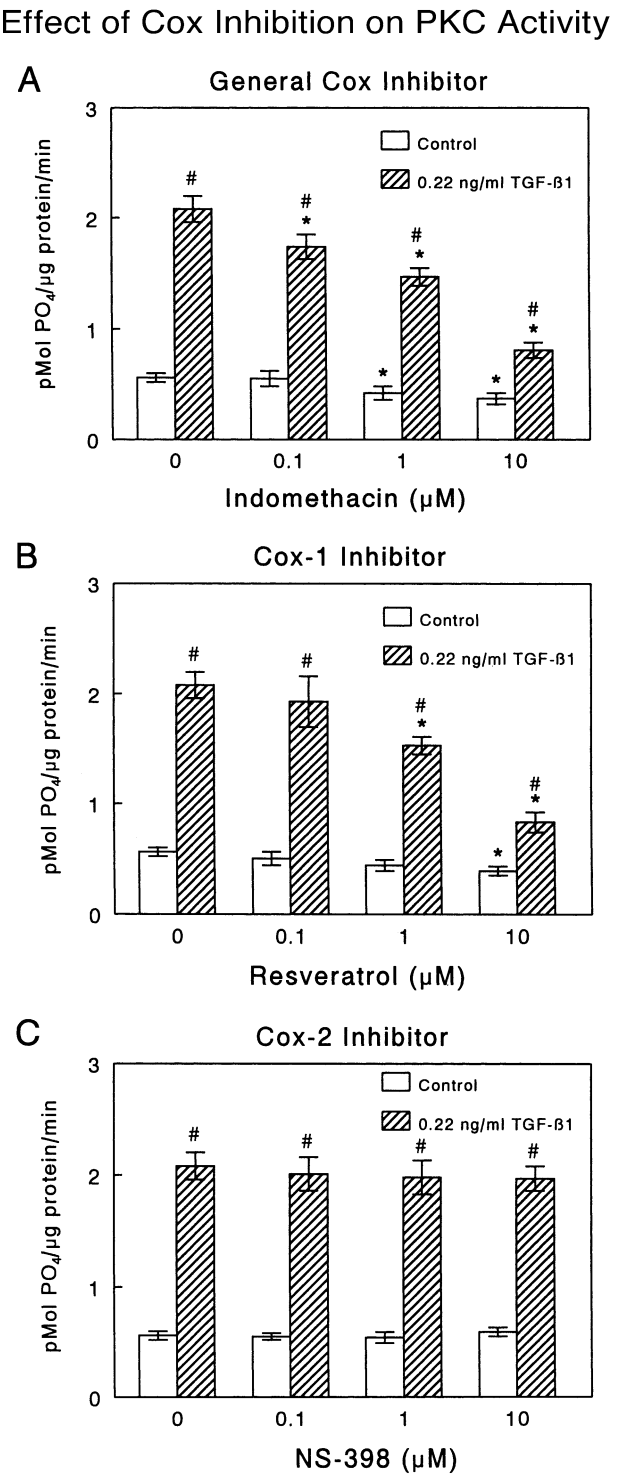

Fig. 5. Effect of cyclooxygenase inhibitors on TGF-β1-induced PKC activity. Confluent growth zone cells were treated for 12 h with control media or 0.22 ng/ml TGF-β1 in the presence and absence of 0.1, 1.0, or 10 μM indomethacin (Panel A), resveratrol (Panel B), or NS-398 (Panel C). At harvest, PKC activity in the cell layer was determined. Values are the mean ± S.E. of six independent cultures. Data are from one of two separate experiments, both with comparable results. *$P<0.05$, vs. no inhibitor; #$P<0.05$, vs. not treated with TGF-β1.

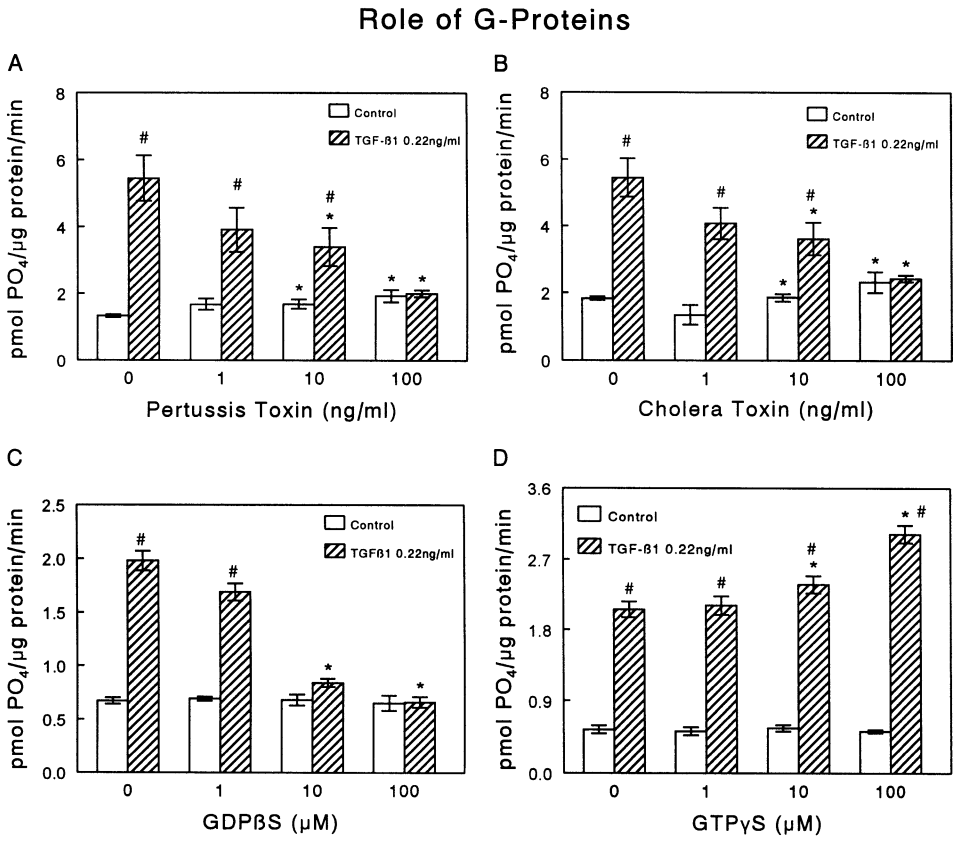

Fig. 6. Role of G-proteins in TGF-β1-induced PKC activity. Confluent growth zone cells were treated for 12 h with control media or 0.22 ng/ml TGF-β1 in the presence and absence of 1, 10, or 100 ng/ml pertussis toxin to inhibit G_i (Panel A) or cholera toxin to inhibit G_s (Panel B). In addition, the general G-protein inhibitor GDPβS (Panel C) or the G-protein activator GTPγS (Panel D) was tested at 1, 10, or 100 μM. At harvest, PKC activity in the cell layer was determined. Values are the mean ± S.E. of six cultures. Data are from one of two separate experiments, both with comparable results. $^{*}P<0.05$, vs. no inhibitor or activator; $^{\#}P<0.05$, vs. not treated with TGF-β1.

but significant, dose-dependent decrease in PKC in control cultures and reduced TGF-β1-induced PKC activity by more than 60% (data not shown).

3.4. Direct effect of TGF-β1 on membrane PKC

The effects of TGF-β1 on PKC are not mediated by a direct effect on preexisting membrane-associated PKC. There was no change in PKC specific activity of isolated matrix vesicles or plasma membranes that were incubated directly with TGF-β1 (data not shown). In addition, TGF-β1 did not elicit a series of membrane-mediated events leading to an increase in preexisting membrane-associated PKC, since specific activity did not change as a function of time (data not shown).

4. Discussion

This study demonstrates that TGF-β1 mediates its effects on proliferation, proteoglycan production, and ALPase activity of GC chondrocytes by a mechanism involving TGF-β receptor II, as has been noted previously by others [12–16]. Blockade of TGF-β1 type II receptor activation with antibodies to the receptor or with soluble receptor blocked the effects of the growth factor on proliferation and ALPase. However, the stimulatory effect of TGF-β1 on [^{35}S]sulfate incorporation was only partially reduced. One possibility is that expression and synthesis of aggrecan, the cartilage proteoglycan core protein, is regulated via the type II receptor [12], whereas glycosaminoglycan synthesis and/or sulfation is only partially regulated in this manner. It is more likely that this parameter is less sensitive to the concentration range used. The type III receptor does not appear to be involved since antibodies to this receptor had no effect.

Although Smad signaling is clearly important in cell response to TGF-β1 [19,67], the results of the present study show that other signaling pathways are involved. The effects of TGF-β1 on the parameters examined here are mediated by PKC-dependent signal transduction. Binding of TGF-β1 to the type II receptor is required for activation of PKC, and inhibition of PKC blocks the stimulatory effects of TGF-β1 on proliferation, proteoglycan sulfation, and ALPase activity.

Not all of the physiological responses of growth zone cells to TGF-β1 are mediated by PKC, however. TGF-β1 caused an increase in PGE_2 production that was independent

of PKC, but depended instead on G-proteins. PKA also plays a role since inhibition of this signaling pathway caused a partial reduction in the stimulatory effect of TGF-β1 on proliferation and ALPase activity. PKA may mediate the response of growth zone cells to TGF-β1 directly or indirectly through modulation of PKC, based on the decrease in TGF-β1-stimulated PKC activity in cultures treated with the PKA inhibitor H-7.

Our study relies on the use of selective inhibitors to resolve the contributions of PKC and PKA signaling to the physiological responses of growth zone cells to TGF-β1. Studies of this kind are complicated by the overlapping sensitivities of these enzymes to the inhibitors used. To overcome this concern, we used concentrations that were optimized for the specific signaling pathway being examined. For example, chelerythrine has an IC_{50} of 0.7 μM for PKC, whereas its IC_{50} for PKA is 170 μM [45]; the highest concentration of chelerythrine used here was 1 μM. Moreover, we verified the contribution of the PKC signaling pathway to the physiological response using alternate inhibitors, again taking care to use doses optimized for PKC. H-7 has been used effectively in other systems to block PKC activity in the same dose range as used in our study [68–70]. H-7 also inhibits other kinases, however, including PKA, PKB, and myosin light chain kinase (MLCK) [69], but the concentrations of H-7 required to do so are considerably greater than the highest dose used in the present study. Similarly, staurosporine can inhibit other serine/threonine kinases as well as some tyrosine kinases. The doses used in our study are appropriate for PKC, however, since staurosporine inhibits PKC with an IC_{50} of 0.7 nM and inhibits PKG with an IC_{50} of 85 nM [71,72]. Inhibition of tyrosine kinases requires 25–50 μM. Finally, we used H-8 to specifically inhibit PKA. Although this approach is cumbersome, it strengthens the interpretation of the result.

The involvement of both signaling pathways is supported by the observations of Osaki et al. [11] using rat articular chondrocytes. These investigators showed that 1 ng/ml TGF-β1 caused a rapid increase in c-*fos* expression, and at 18–24 h, [^{3}H]thymidine incorporation was increased. When the articular chondrocytes were treated with 50 μM H-7, the stimulatory effect of TGF-β1 on DNA synthesis was blocked. The levels of H-7 used exceeded the IC_{50} for PKA (6.0 μM) as well as the IC_{50} for PKC (3.0 μM), thereby implicating both kinases in the mechanism.

These observations also support the hypothesis that different signaling pathways mediate the effects of TGF-β1 in resting zone and growth zone chondrocytes. In the less mature resting zone cells, neither TGF-β1-dependent proliferation nor ALPase requires PKC [29], whereas both parameters are mediated by PKC in growth zone cells. Moreover, part of the effect of TGF-β1 on proliferation and ALPase activity in growth zone cells is mediated by PKA.

The mechanism of PKC activation by TGF-β1 in growth zone cells differs from the classical pathway of PKC activation, in which PLC or PLD act on membrane phospholipids to generate the second messenger, diacylglycerol, which then binds and activates PKC [73]. In the present study, we show that diacylglycerol is not involved in the TGF-β1-dependent activation of PKC since exogenous diacylglycerol had no effect on TGF-β1-stimulated PKC. Moreover, increasing endogenous diacylglycerol by inhibiting DAG kinase also failed to affect PKC in response to TGF-β1. In general, diacylglycerol is produced as a rapid response to receptor activation, but TGF-β1-dependent PKC is a delayed response, implicating a regulatory cascade. Our results indicate that no component of the cascade mediates its effects via PLC or diacylglycerol, since DOG or the DAG kinase inhibitor were present throughout the 12-h incubation. This supports our previous observation showing that the stimulatory effect of TGF-β1 on PKC activity does not involve PLC, based on the inhibition of PLC activity with the PI-PLC inhibitor U73122 [20]. It also rules out the possibility of a role for diacylglycerol produced through a PLD-dependent mechanism.

As noted previously for resting zone cells [22] and renal tubular epithelial cells [74], G-proteins also mediate the effects of TGF-β1 on PKC in growth zone chondrocytes, since the general inhibitor GDPβS blocked PKC stimulation in response to the growth factor. Both G_i and G_s appear to be involved. TGF-β1 stimulation of PKC can be blocked by pertussis toxin, which inhibits G_i, leading to an increase in cAMP. In addition, TGF-β1 stimulation is blocked by choleratoxin, indicating that G_s also plays a role. Choleratoxin activates the α_s subunit, so that cAMP is continuously being produced [75]. This again implicates PKA in the mechanism, but suggests that cAMP levels are important variables in mediating the response to the growth factor.

Part of the effect of TGF-β1 on PKC in growth zone cells is mediated by the PLA_2 pathway. Arachidonic acid, the product of PLA_2 action, by itself increased PKC activity in the cells, and at the highest concentration used, was additive with TGF-β1. This differs from our previous observations using resting zone cells, where activation of PLA_2 inhibits the stimulatory effect of TGF-β1 on PKC [29]. In addition, exogenous arachidonic acid inhibits PKC activity in resting zone cells [30], underscoring the role of cell maturation in the mechanism.

Arachidonic acid is a known activator of PKC, acting in part through direct nongenomic mechanisms [76,77]. It also may increase PKC by activating gene expression via PPAR receptors [78]. Arachidonic acid is also the substrate for cyclooxygenase, resulting in prostaglandin production. TGF-β1 stimulated PGE_2 release into the conditioned media at the same dose and time that elicited maximal increases in PKC activity, suggesting that PGE_2 might mediate the effect of the growth factor on PKC. Exogenous PGE_2 stimulated PKC, as reported previously [47], but the increase was less than that achieved by TGF-β1 alone. This suggests that PGE_2-dependent mechanisms may be involved, but are not sufficient to elicit the complete PKC response. Our results indicate that prostaglandins other than PGE_2 play a role

since the general cyclooxygenase inhibitor indomethacin blocks the TGF-β1-dependent increase in PKC.

It is likely that the regulatory step is at PLA_2 and production of arachidonic acid. Previously, we have reported that TGF-β1 decreases PLA_2 in plasma membranes and matrix vesicles in cultures treated for 24 h with the growth factor [6]. It is clear from the present study, however, that TGF-β1 increases PGE_2 production. Since PGE_2 is derived from arachidonic acid, these results suggest that TGF-β1 may cause an increase in PLA_2 activity at an earlier time point, resulting in a greater pool of arachidonic acid. The increase in PGE_2 levels is not due to an increased rate of metabolism of arachidonic acid because only inhibition of Cox-1, the constitutive form of cyclooxygenase, blocked the TGF-β1-dependent effect. Inhibition of Cox-1 mimicked the effect of indomethacin, which inhibits both Cox-1 and Cox-2. In contrast, inhibition of Cox-2 had no effect on TGF-β1-dependent PKC.

Exogenous PGE_2 has multiple effects on growth zone cells, promoting differentiation and anabolic responses via cAMP production and PKC activity [49]. Prostaglandins exert their effects on cells via EP receptors, which are G-protein-coupled, potentially accounting for the inhibition in PKC activity when G-proteins are inhibited. Growth zone chondrocytes express both EP1 and EP2, as well as a variant of EP1, EP1v [51]. Previous studies showed that PGE_2 directly increased PKC via EP1. However, EP1 signals through PLCβ [52], and the effect of TGF-β1 does not involve PLC. PGE_2 can also increase PKC indirectly through EP2 receptor activation by signaling through cAMP production and PKA [52], although this mechanism is not used in control cultures of growth zone cells treated with EP2 agonists [51]. This pathway may contribute to PKC activation in response to TGF-β1, however, since inhibition of PKA with H-8 caused a reduction in TGF-β1-induced PKC activity. Moreover, H-8 reduced the TGF-β1-dependent increases in [^{3}H]thymidine incorporation and ALPase activity.

In summary, the results of this study show that the effects of TGF-β1 on growth zone chondrocytes are mediated by the type II receptor involving at least three separate pathways: via PKC, PLA_2/arachidonic acid/prostaglandin, and PKA. Additionally, there is cross-talk between these pathways (see Fig. 7). In this schematic diagram, TGF-β1 binds to its type II receptor, which binds to the type I receptor. TGF-β1 type I receptor activates Smad proteins and also activates a G-protein-dependent cascade, altering the activity of PLA_2, which produces arachidonic acid. Depending upon the time it is measured, PLA_2 activity may either be activated or inhibited, as indicated by the "±". Arachidonic acid is metabolized by cyclooxygenase to prostaglandins, which bind to EP1 and EP2 receptors on the growth zone chondrocyte cell surface [51]. EP1 signaling stimulates PKC activity, while EP2 signaling increases cAMP, which activates PKA. This suggests that TGF-β1 signals through PKC and PKA to increase ALPase activity and to stimulate

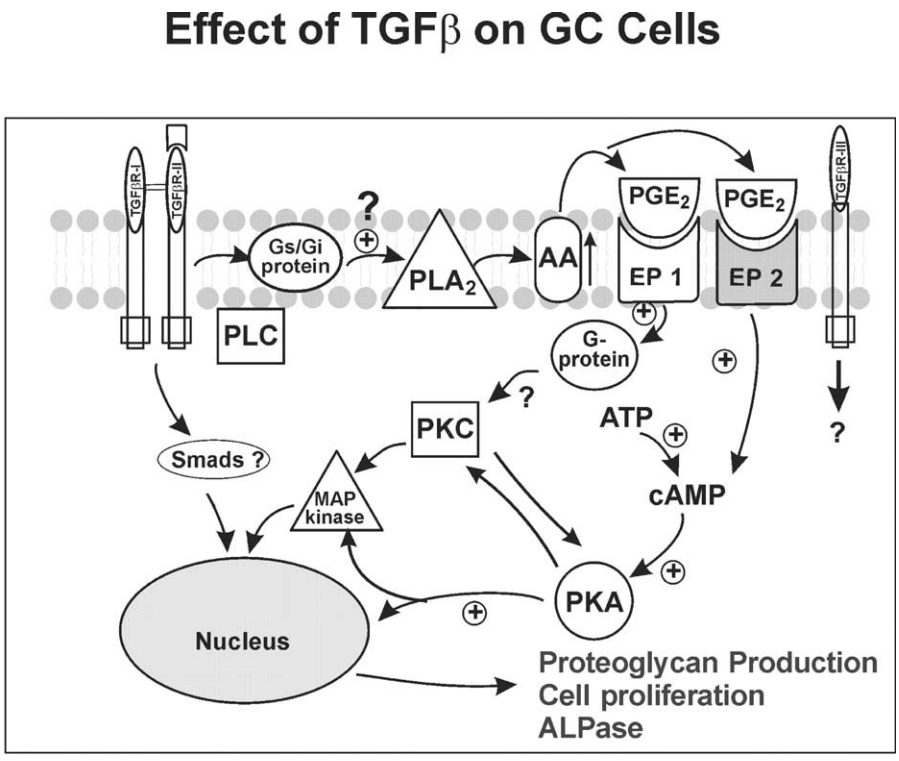

Fig. 7. Mechanisms of TGF-β1 action in growth zone chondrocytes. Transforming growth factor-β1 (TGFβ1); phospholipase A_2 (PLA_2); arachidonic acid (AA); prostaglandin E_2 (PGE_2); alkaline phosphatase (ALPase); protein kinase A (PKA); protein kinase C (PKC); "−"=inhibition, "+"=stimulation, "±"=inhibition or stimulation, time-dependent. See last paragraph of Discussion for additional details.

proliferation and through PKC to stimulate proteoglycan production. Therefore, the regulation of the PKC pathway, the PKA pathway and the PLA_2/arachidonic acid/prostaglandin pathway by TGF-β1 is essential for its role in the proliferation and differentiation of growth plate chondrocytes.

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